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PRINCIPAL INVESTIGATOR: Thomas J. Sick, Ph.D.

CONTRACTING ORGANIZATION: University of Miami
Miami, Florida 33136

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13. ABSTRACT (Maximum 200 Words) Mitochondria normally function to provide sources of energy for vital cellular functions. However, under stressful conditions these organelles may trigger events that lead eventually to cell death. Thus, mitochondria have been implicated as major contributors to neuronal death in a variety of neurodegenerative disorders. In this report we describe functional changes in mitochondria measured in living brain tissue as a result of exposure to toxins thought to contribute to neurodegeneration. Mitochondrial NADH levels were shown to increase following treatment with 1-methyl-4-phenylpyridium (MPP ⁺) at times when neuronal electrophysiology was impaired. In addition, MPP ⁺ appeared to cause oxidation of cytochrome b, providing further support that this toxin inhibits Mitochondrial complex I. 3-nitropropionic acid (3-NP) also resulted in blockade of neuronal electrical activity but had minimal effects on mitochondrial NADH and redox activity of respiratory chain cytochromes. Both toxins produced delayed and selective cell death in hippocampal subfield CA1. Neurons in this subfield have been shown previously to be selectively vulnerable to hypoxia/ischemia. The data reported here suggest that these neurons may also be selectively vulnerable to mitochondrial toxins.				
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FOREWORD

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Tony Lau

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Introduction

The work funded by this contract is directed toward understanding the functional changes in brain mitochondria associated with exposure to mitochondrial toxins, and to determine whether these changes are compatible with neuronal survival, acute neuronal dysfunction or known signals of delayed or apoptotic cell death. The goals are: 1) to first characterize toxin-induced alterations of respiratory chain redox status and mitochondrial membrane potential in intact functioning neuronal populations, and to assess the reversibility of acute toxin exposure in terms of mitochondrial and neuronal function; 2) to examine the role of specific intracellular mediators of mitochondrial and neuronal dysfunction including release of mitochondrial cytochrome *c*, calcium overload, reactive oxygen species (ROS), and mitochondrial permeability transition. Proposed experiments will utilize brain slice preparations for monitoring mitochondrial redox activities, mitochondrial membrane potential ($\Delta\psi_m$) and electrical function. Rapid scanning spectrophotometry and spectrofluorometry will be used to assess mitochondrial function, while microelectrode electrophysiology will be used to evaluate neuronal function.

Body

As outlined by the Statement of Work for this contract, we have begun evaluation of the mitochondrial redox consequences of brain slice exposure to mitochondrial toxins. To date, we have evaluated exposure to 1-methyl-4-phenylpyridium (MPP⁺), the active toxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a putative Parkinson's Disease toxin (Mari and Bodis-Wollner, 1997). We have also tested exposure of brain slices to 3-nitropropionic acid (3-NP), a toxin used experimentally to induce Huntington's Disease-like symptoms in animals (Ferrante et al., 1997).

First year experiments focussed on redox changes in mitochondrial nicotinamide adenine dinucleotide (NADH/NAD⁺) measured spectrofluorometrically, and mitochondrial cytochromes *b*, *c*, and *a₃* measured spectrophotometrically. We also simultaneously evaluated changes in neuronal electrophysiology. The first year of funding was also used to develop brain slice cultures suitable for long term evaluation of neuronal viability. These cultures are a valuable complement to our acute brain slices methods that will help establish a link between acute mitochondrial dysfunction and both apoptotic or necrotic neuronal death. Finally, our laboratory has successfully developed reliable assays of cytosolic cytochrome *c* content using Western blot analysis and cell fractionation techniques for use in small tissue samples such as brain slices or brain slice cultures. These experiments are described in detail below.

Mitochondrial Redox Activity After Toxin Exposure

1) General Method

Hippocampal slices were prepared from male Wistar rats weighing 250-300 g. The slices were placed in a recording chamber specially designed for simultaneous optical measurements of mitochondrial function and electrophysiological measurements of neuronal activity. The slices were initially incubated in artificial cerebrospinal fluid (ACSF) before switching to ACSF containing toxins. Control slices were incubated continuously in ACSF devoid of any toxin.

NADH/NAD⁺ was measured spectrofluorometrically. This method takes advantage of the fact that the reduced form (NADH) of this respiratory chain substrate fluoresces while the

oxidized form (NAD^+) does not. Fluorescence was measured by illuminating brain slices with 337 nm excitation light and measuring NADH fluorescence emission by rapid scanning spectrofluorometry (see Rosenthal et al., 1995). Mitochondrial NADH levels are important because NADH is the main source of reducing equivalents (electrons) required for respiratory chain function and the production of the cellular energy source adenosine triphosphate (ATP).

Redox activity of mitochondrial cytochromes b, c, and a_3 were measured spectrophotometrically by transilluminating hippocampal slices with white light and measuring absorption at wavelengths characteristic of individual respiratory chain pigments (LaManna et al., 1985, 1987). This procedure takes advantage of the fact the reduced forms of these respiratory chain pigments absorb light while the oxidized forms do not.

Electrophysiological function was evaluated by measuring electrical responses of hippocampal pyramidal cells in subfield CA1 following electrical stimulation of excitatory afferent fibers (Schaffer collaterals) in subfield CA3. Such stimulation results in a population evoked field potential in CA1 consisting of a fast field excitatory post synaptic potential and population action potential. Examples of these responses and the effect of toxin exposure are shown below.

2) MPP^+ Exposure

Exposure of hippocampal slices to MPP^+ significantly impaired electrical function. Synaptic transmission between Schaffer collaterals and pyramidal neurons in subfield CA1 was depressed after approximately 1 hr of exposure to 100 μM MPP^+ . Examples of evoked potentials recorded during control periods and after toxin exposure are shown in figure 1 (all figures in appendix). Inhibition of synaptic transmission following exposure to 100 μM MPP^+ was complete and similar to that found following anoxia (figure 4).

Simultaneous measurements of NADH fluorescence indicated that exposure to MPP^+ resulted in increased mitochondrial NADH. Examples of NADH emission spectra acquired during control conditions and following exposure to MPP^+ are shown in figure 4. The data are summarized in figures 3 and 4 which show the time course and average changes in both NADH levels and evoked potential activity following exposure to 100 μM MPP^+ . Also shown is the response to anoxia (nitrogen exposure) which resulted in maximum NADH fluorescence and complete inhibition of electrical activity.

Increased NADH levels can only occur if either 1) mitochondrial NADH production through the citric acid cycle increased, or 2) NADH oxidation by the respiratory chain is inhibited. Increased production of mitochondrial NADH is not compatible with the observed inhibition of electrical activity. The data, however, are consistent with other reports (refs) that MPP^+ inhibits respiratory chain complex I which supplies NADH to the respiratory chain.

To determine the effect of MPP^+ exposure on redox activity of mitochondrial cytochromes, absorption difference spectra were acquired that compared control and toxin treatment. Changes in redox activity of mitochondrial cytochromes may be observed as absorption changes at wavelengths characteristic of each respiratory chain pigment. An example of a difference spectrum comparing control and anoxic conditions in a hippocampal slice is shown in figure 5. This figure shows the relative spectral position of absorption maxima for cytochromes b, c, and a_3 . An upward going peak signals increased absorption and an increased reduced/oxidized redox state of the respiratory chain pigment.

Figure 6 shows the effect of MPP^+ exposure on absorption changes of respiratory chain pigments in a hippocampal slice. Exposure of the hippocampal slice to 100 μM MPP^+ resulted in a decrease in absorption near 430 nm and also near 565 nm. Decreased absorption at these wavelengths signals a change toward oxidation of cytochrome b following toxin exposure. No changes were observed at wavelengths characteristic of cytochromes c or a, a_3 . This result is consistent with inhibition of substrate supply to the respiratory chain. Since cytochrome b is partially reduced under control conditions, exposure to MPP^+ resulted in a decrease in its absorption. In contrast, cytochromes c and a, a_3 are nearly fully oxidized under control conditions and thus no further oxidation (or decrease in absorption) could be observed at wavelengths characteristic of these respiratory chain pigments.

3) 3-NP Exposure

Similar experiments were conducted to test the effects of 3-NP on electrophysiology and mitochondrial function in hippocampal slices. Evoked potentials were recorded in hippocampal subfield CA1 following electrical stimulation of the Schaffer collaterals, while NADH fluorescence was monitored simultaneously. The results are summarized in figures 7 and 8. Exposure of hippocampal slices to 1 mM 3-NP resulted in a small increase in NADH fluorescence, but not as robust as that observed following exposure to MPP^+ . However, similar to MPP^+ , 3-NP treatment inhibited synaptic transmission. There were no consistent effects of 3-NP on redox activity of mitochondrial cytochromes b, c, or a, a_3 as determined by spectrophotometric measurements of absorption changes.

In contrast to MPP^+ , no consistent changes in mitochondrial redox activity were observed following treatment of hippocampal slices with 3-NP (data not shown). This may indicate that under resting conditions, most of the respiratory chain electron flow is provided through complex I (inhibited by MPP^+) than through complex II (inhibited by 3-NP). The lesser effects of 3-NP on mitochondrial NADH levels (see above) support this conclusion.

Cell Death After Toxin Exposure

The main goal of research funded by this contract is to examine acute changes in brain mitochondrial function after toxin exposure and to determine whether these changes predict and contribute to neuronal dysfunction or neuronal death. The acute hippocampal slice preparation is not well suited for examining long-term changes in neuronal function or for examining cell death that may occur days to weeks after toxin exposure. Our laboratory has now successfully employed the organotypic slice culture model to examine more chronic neuronal damage. These preparations consist of hippocampal slices placed in culture for approximately 2 week prior to toxin exposure. Figure 9 shows an image of a 2 wk old hippocampal culture stained with the immunohistochemical marker NEUN which is specific for forebrain neurons. The stain clearly shows intact neurons characteristic of hippocampal subfields CA3 and CA1.

To assess cell death, hippocampal slices cultures were exposed for 3 hr to either 400 μM MPP^+ or 1 mM 3-NP. Cell death was assessed with the fluorescent indicator propidium iodide (PI). PI shows intense red fluorescence when it reacts with nuclear DNA. PI does not penetrate intact cell or nuclear membranes and thus does not fluoresce when cells are viable.

1) MPP^+ Exposure of Hippocampal Cultures

Figure 10 shows an example of the effect of MPP^+ on cell death in a hippocampal slice culture. This 2-wk old slice culture was incubated in 400 μM MPP^+ for 3 hr and then assessed for cell death 4 days following exposure. The upper image is a brightfield image of the hippocampal slice culture that shows the approximate position of CA3 and CA1 pyramidal subfields. The lower image shows intense PI fluorescence principally confined to the CA1 hippocampal subfield. This data indicates that MPP^+ exposure results in selective cell death of hippocampal neurons. In this respect, the toxin acts similarly to hypoxia/ischemia which also selectively damage cells in hippocampal subfield CA1.

2) 3-NP Exposure of Hippocampal Cultures

Figure 11 shows an example of the effect of 3-NP on cell death in a hippocampal slice culture. This 2-wk old culture was exposed to 1 mM 3-NP for 3 hr and then tested for cell death 2 days following exposure. Again, the upper image is a brightfield image of the slice culture and the lower image shows PI fluorescence. As with MPP^+ , 3-NP produced cell death that was largely confined to the CA1 hippocampal subfield. This data demonstrates that yet another distinct form of mitochondrial inhibition results in selective hippocampal cell damage.

As outlined in the Statement of Work for this contract, future studies will attempt to determine the mechanism by which mitochondrial dysfunction leads to neuronal death. We will evaluate the role of intracellular calcium ion elevation following toxin exposure and the role of oxygen free radicals. Future studies will also attempt to determine whether release of mitochondrial constituents such as cytochrome c contributes to cell death following toxin exposure.

Key Research Accomplishments

- 1) The Parkinsonian toxin 1-methyl-4-phenylpyridium (MPP^+) blocked synaptic transmission within 1 hr in hippocampal slices.
- 2) MPP^+ increased levels of mitochondrial NADH and oxidized cytochrome b at the time synaptic electrophysiology was impaired.
- 3) MPP^+ -treated hippocampal slice cultures showed fluorescence staining in hippocampal subfield CA1 2 days after treatment indicating selective cell death.
- 4) The Huntington's toxin 3-nitropropionic acid (3-NP), blocked synaptic transmission within 1 hr in hippocampal slices.
- 5) 3-NP did not produce marked changes in redox state of mitochondrial respiratory chain components.
- 6) 3-NP produced selective cell death in the CA1 subfield of hippocampal slice cultures.

Reportable Outcomes

NONE AT PRESENT

Conclusions

The results reported here indicate that chemical toxins may specifically target brain mitochondria and through this mechanism affect brain function and perhaps also promote cell death. The data suggest that a first signal of toxin-induced mitochondrial damage may be inhibition of electron transfer through the respiratory chain. In this way certain mitochondrial toxins may act similarly to hypoxia or hypoglycemia. Our first year data showing selective

vulnerability of specific hippocampal neuronal populations supports this conclusion. Future studies outlined in the Statement of Work for this research program will specifically target mechanisms by which inhibition of mitochondrial function promotes functional damage and cell death. Knowledge of the time course of the progression from mitochondrial dysfunction to irreversible cell death and the pathways involved in this progression is a necessary step for designing effective therapeutic interventions.

The importance of this research stems not only from the basic knowledge provided about mechanisms of toxin-induced brain damage, but also the need to develop new diagnostic procedures for assessing toxin exposure. The data show that measurements of mitochondrial function can be accomplished in intact functioning brain tissue. This offers the possibility that signals of mitochondrial function may be used clinically for early diagnosis of exposure and perhaps assessment of the state of mitochondrial damage. Such signals of mitochondrial function may also be valuable for determining the effectiveness of therapeutic interventions.

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Appendix

Figure Legends

Figure 1. Effect of 100 μM MPP^+ on evoked field potentials in a hippocampal slice. Field potentials (negative deflections following stimulus artifact) were elicited in subfield CA1 by electrical stimulation of the Schaffer collaterals. Field potentials were abolished within 60 min of treatment with MPP^+ .

Figure 2. Examples of NADH emission spectra acquired from a hippocampal slice during control conditions, after treatment with 100 μM MPP^+ , and after exposure to nitrogen. NADH shows broad fluorescence emission peaking at approximately 450 nm. Fluorescence was measured from subfield CA1 following excitation from a pulsed nitrogen laser (337 nm). Spectra are averages of 5 consecutive 1 sec acquisition periods. MPP^+ resulted in an increase in NADH fluorescence within 30 min.

Figure 3. Average changes in NADH fluorescence emission in untreated slices and in slices treated with 100 μM MPP^+ . NADH emission is expressed as the percent of the first control value. (Control $n = 5$, $\text{MPP}^+ n = 8$).

Figure 4. Average amplitudes of evoked potentials recorded in subfield CA1 of hippocampal slices treated with 100 μM MPP^+ or slices exposed to ACSF without drug. (Control $n = 5$, $\text{MPP}^+ n = 5$).

Figure 5. Example of an absorption spectrum recorded from a hippocampal slice. This spectrum is a "difference" spectrum comparing the changes in absorption during the transition from normoxia to anoxia ($\log(\text{anoxia}/\text{normoxia})$). This difference spectrum shows some of the characteristic absorption peaks for the mitochondrial respiratory chain pigments.

Figure 6. Absorption difference spectrum comparing control and MPP^+ treatment of a hippocampal slice. The large decrease in absorption at approximately 430 nm and that at approximately 565 nm signals a shift toward oxidation of cytochrome b.

Figure 7. Average changes in NADH fluorescence in hippocampal slices following treatment with 1 mM 3-NP or in slices devoid of drug. Values are expressed as the percent of the control pretreatment level of fluorescence. (Control $n = 5$, 3-NP $n = 8$)

Figure 8. Average amplitudes of evoked potentials recorded in subfield CA1 of control hippocampal slices or hippocampal slices treated with 1 mM 3-NP. (Control $n = 5$, 3-NP $n = 8$)

Figure 9. Brightfield image of an organotypic hippocampal slice culture maintained in culture for 2 weeks. Neurons were stained with the neuron specific marker NEUN. Clearly visible are the neurons in subfields CA1 and CA3.

Figure 10. Upper Image: brightfield image of an organotypic slice culture prior to treatment with MPP^+ showing the relative position of subfields CA1 and CA3. Lower Image: fluorescence image of the same slice stained with propidium iodide 4 days after a 3 hr treatment with 400 μM MPP^+ . Intense propidium iodide fluorescence indicates the presence of dead cells.

Figure 11. Upper Image: brightfield image of an organotypic slice culture prior to treatment with 3-NP showing the relative positions of subfields CA1 and CA3. Lower Image: fluorescence image of the same slice stained with propidium iodide 2 days after a 3 hr treatment with 2 mM 3-NP.

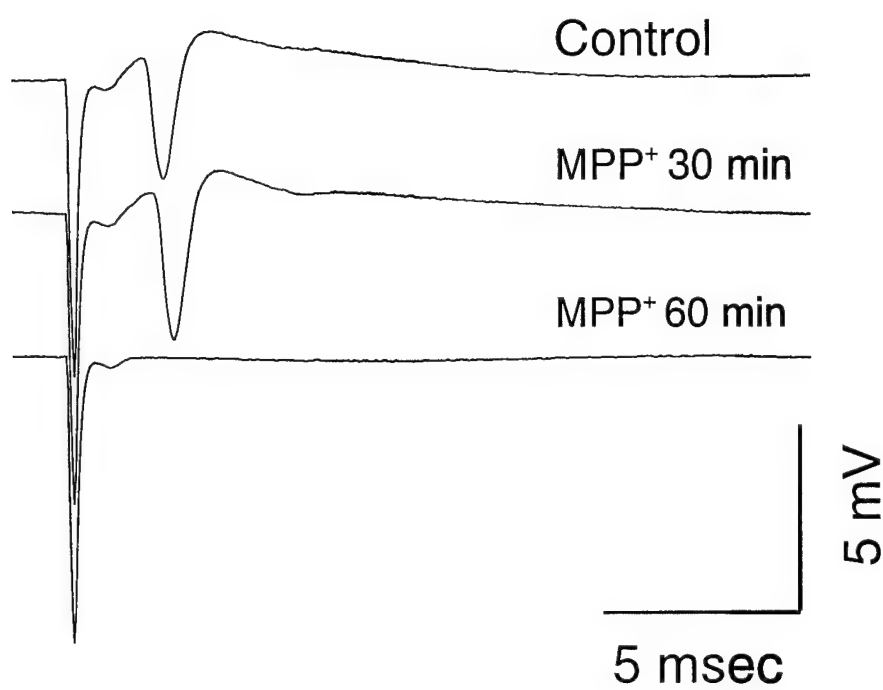


Figure 1

100 μ M MPP⁺

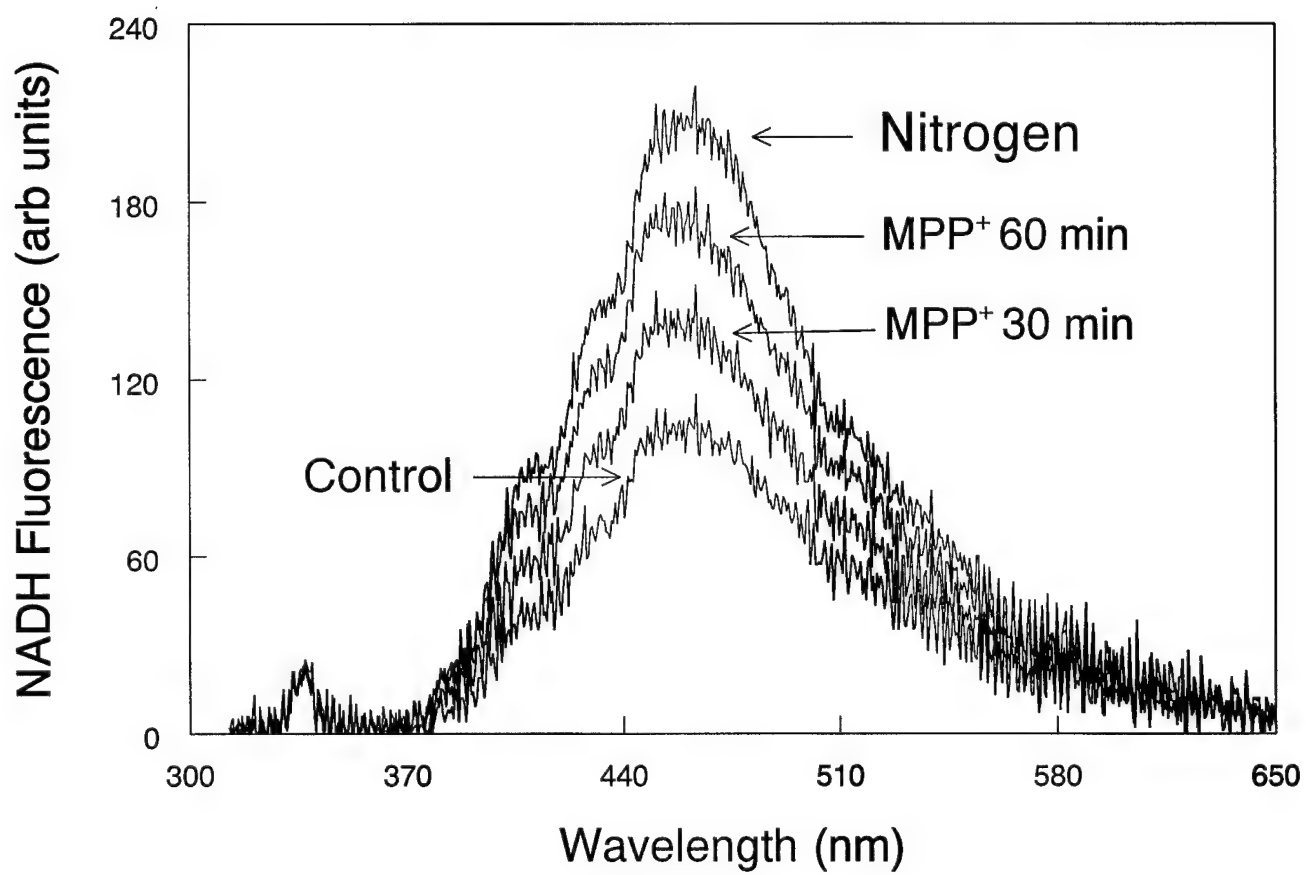


Figure 2

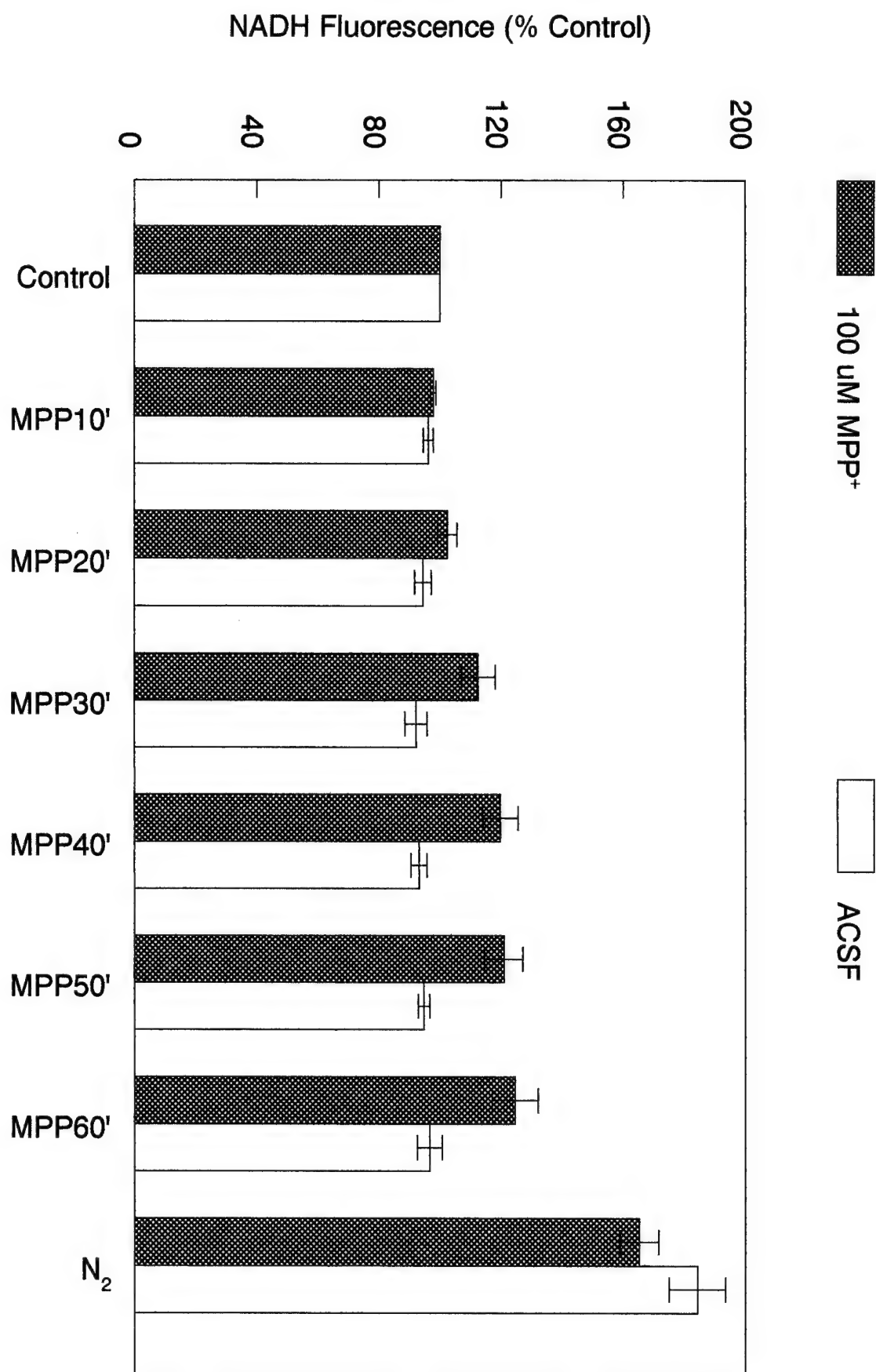


Figure 3

Evoked Potential Amplitude (mV)

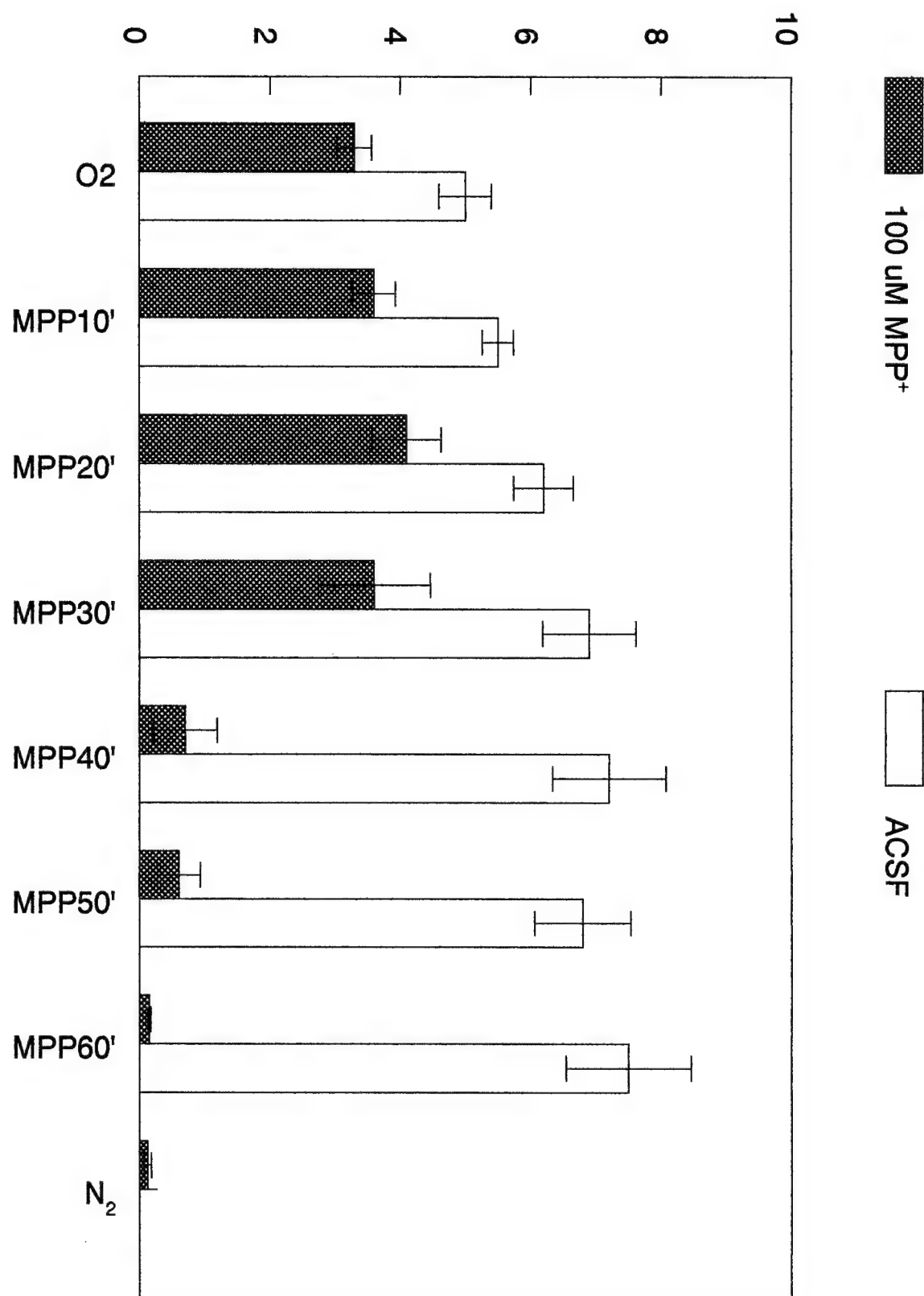
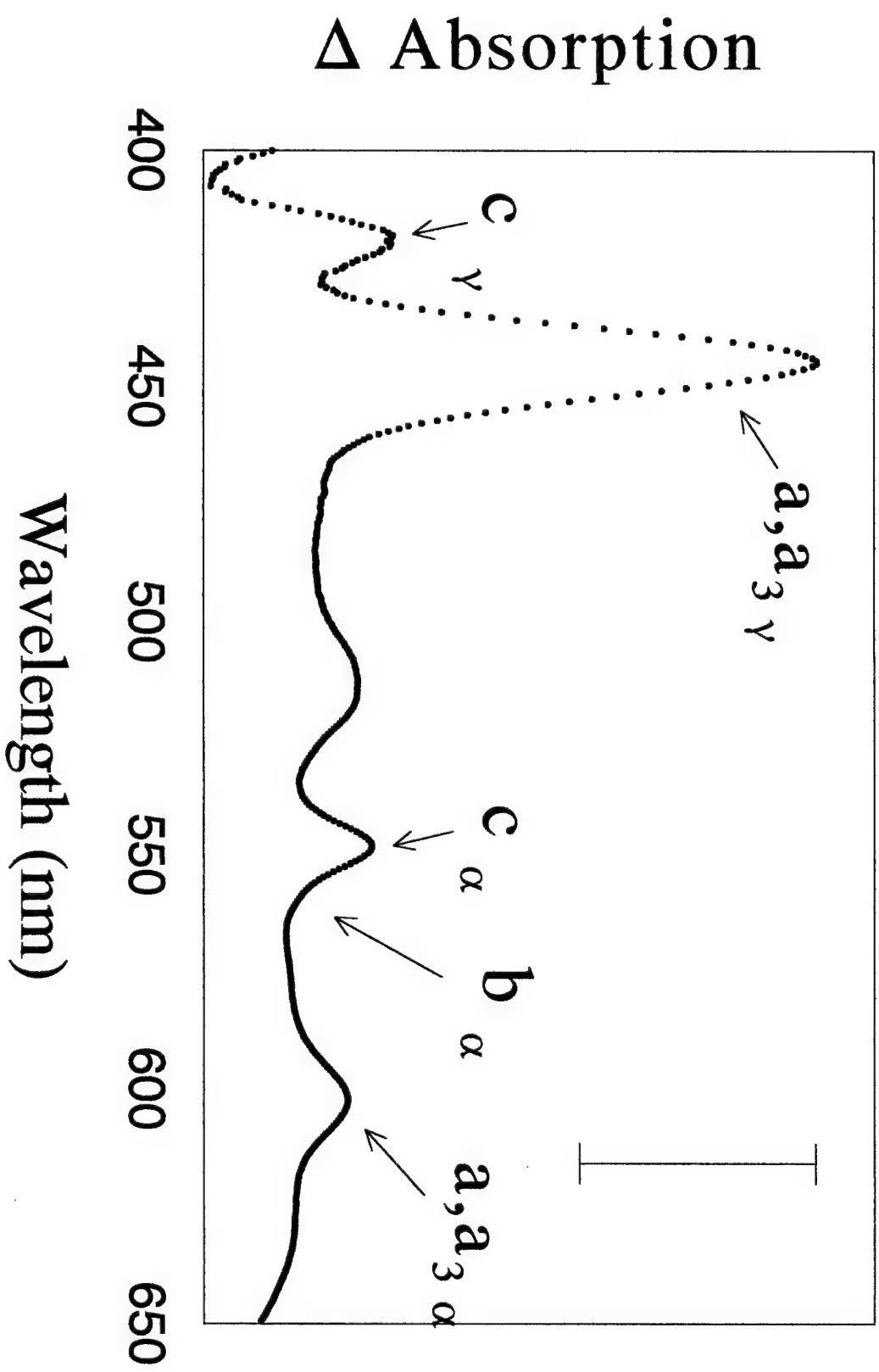


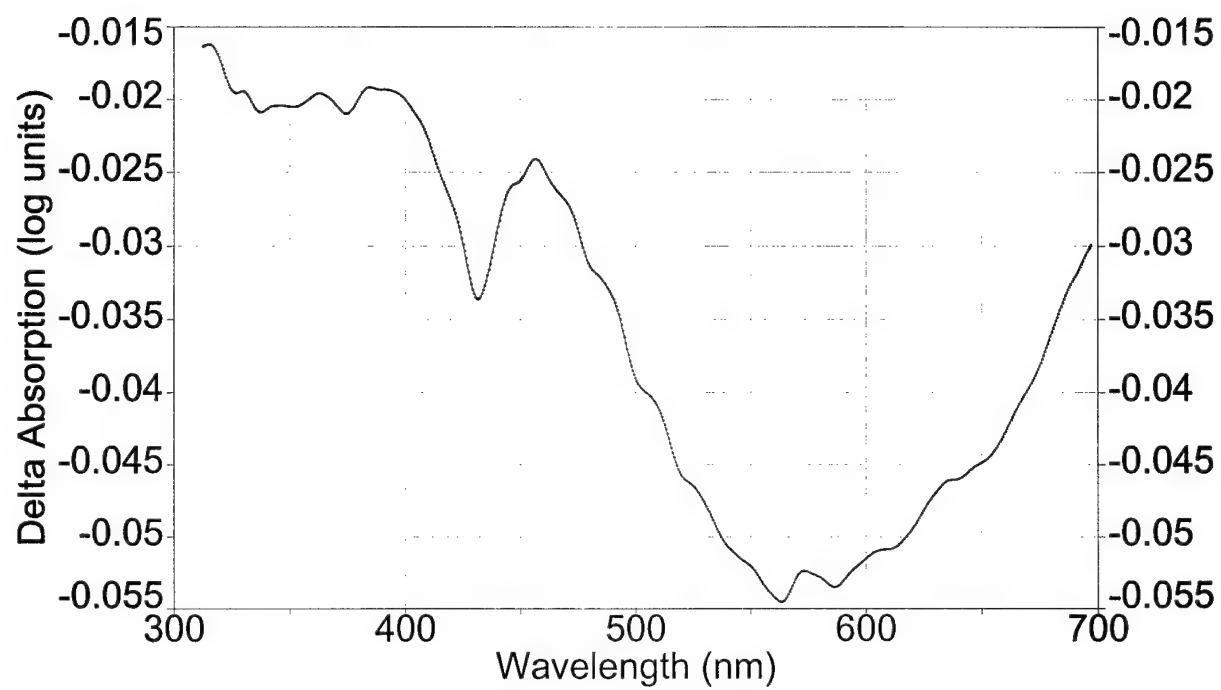
Figure 4



Wavelength (nm)

Figure 5

Figure 6



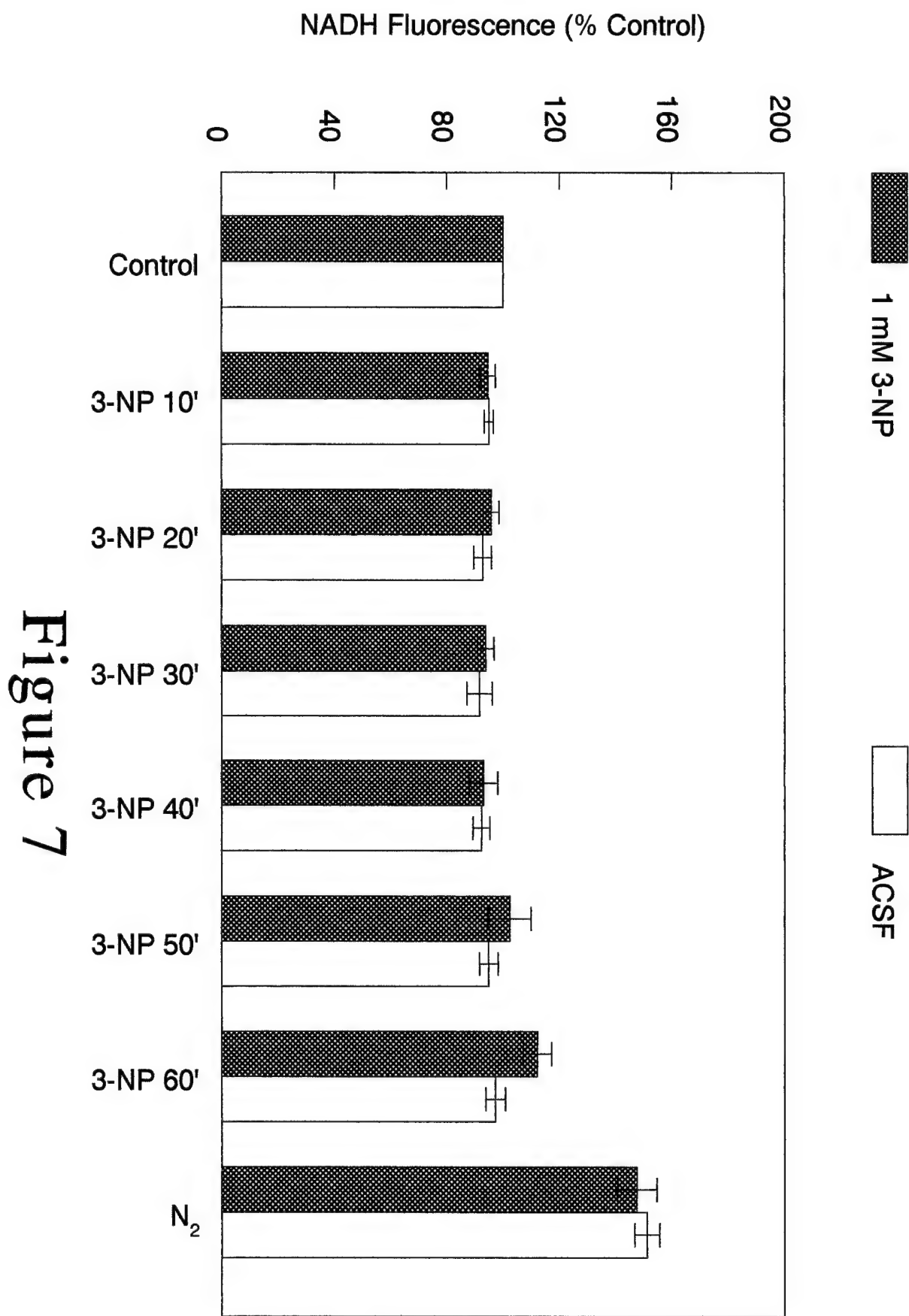


Figure 7

Evoked Potential Amplitude (mV)

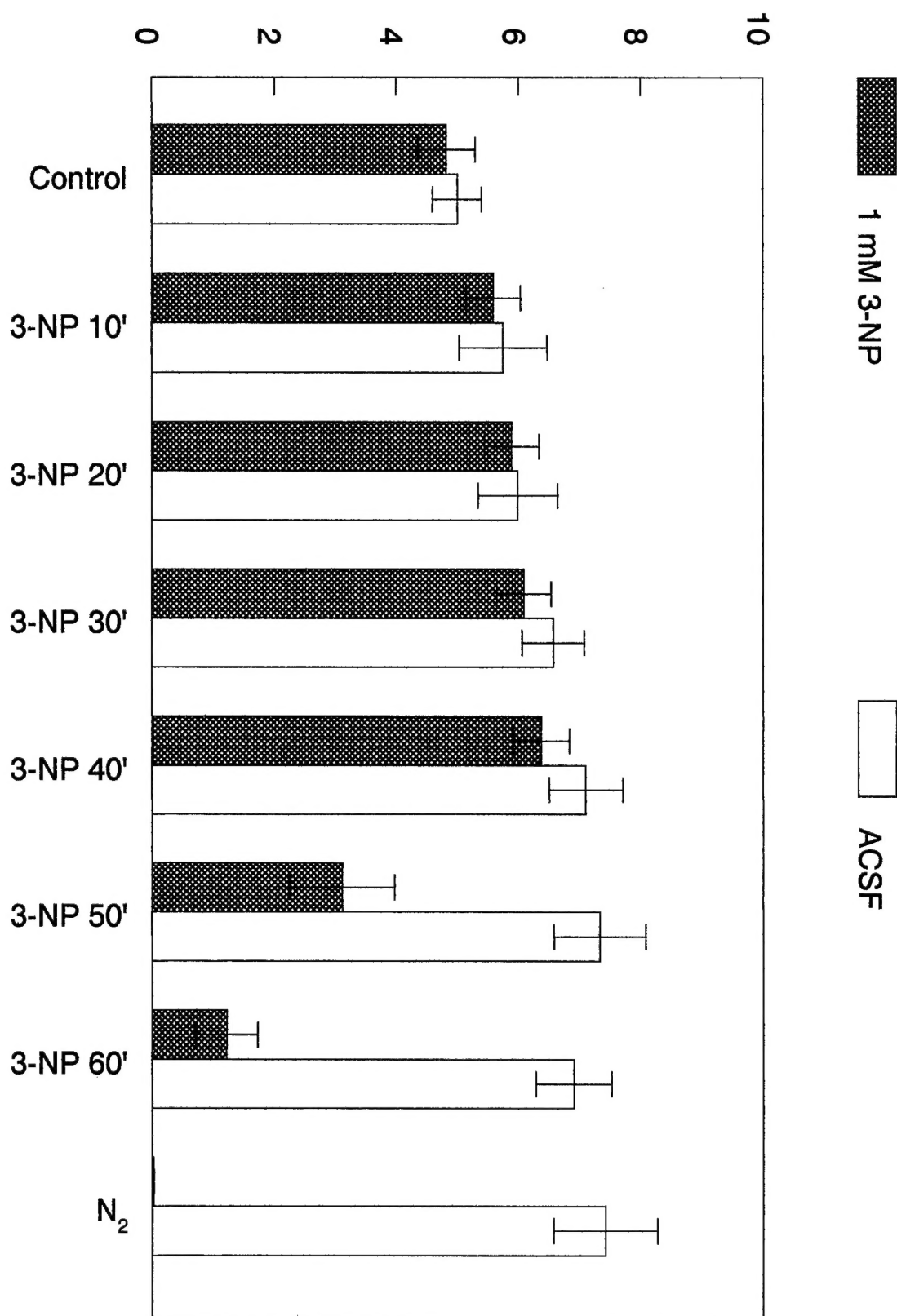


Figure 8

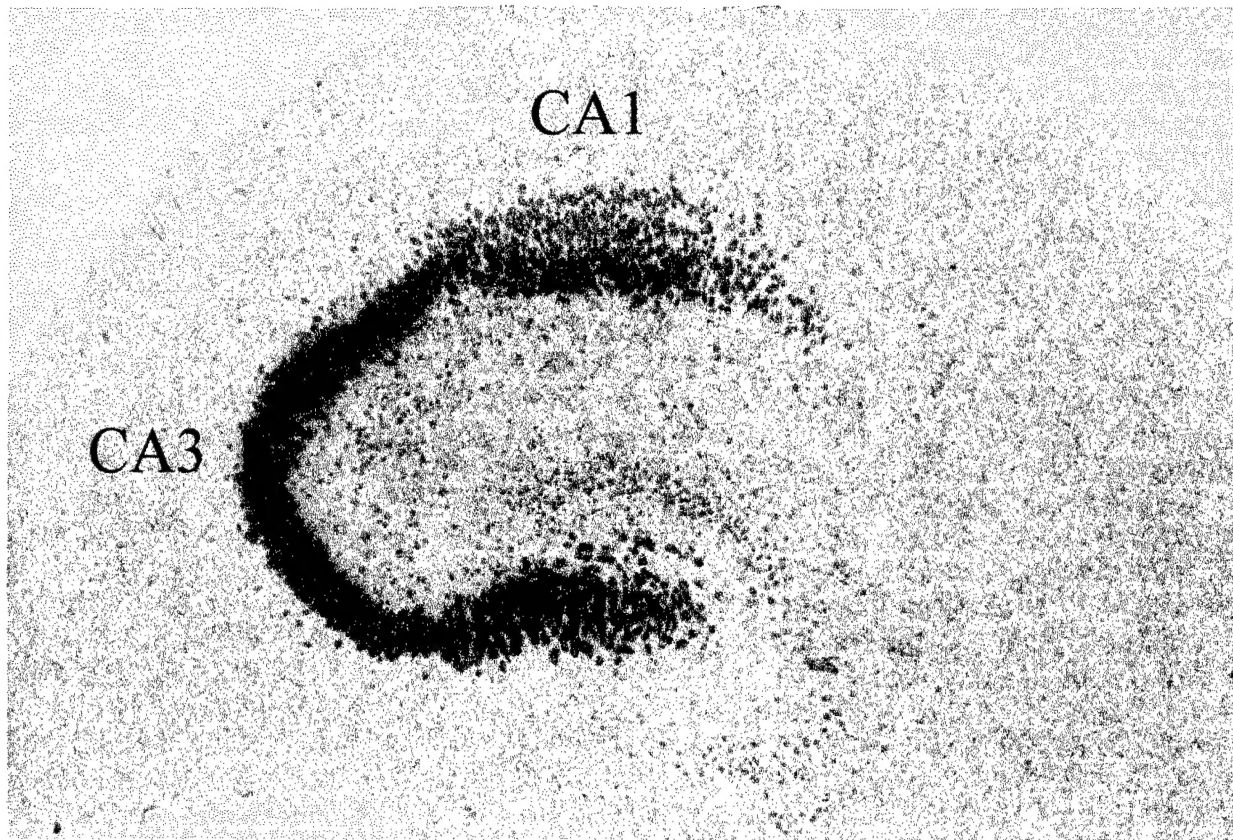


Figure 9

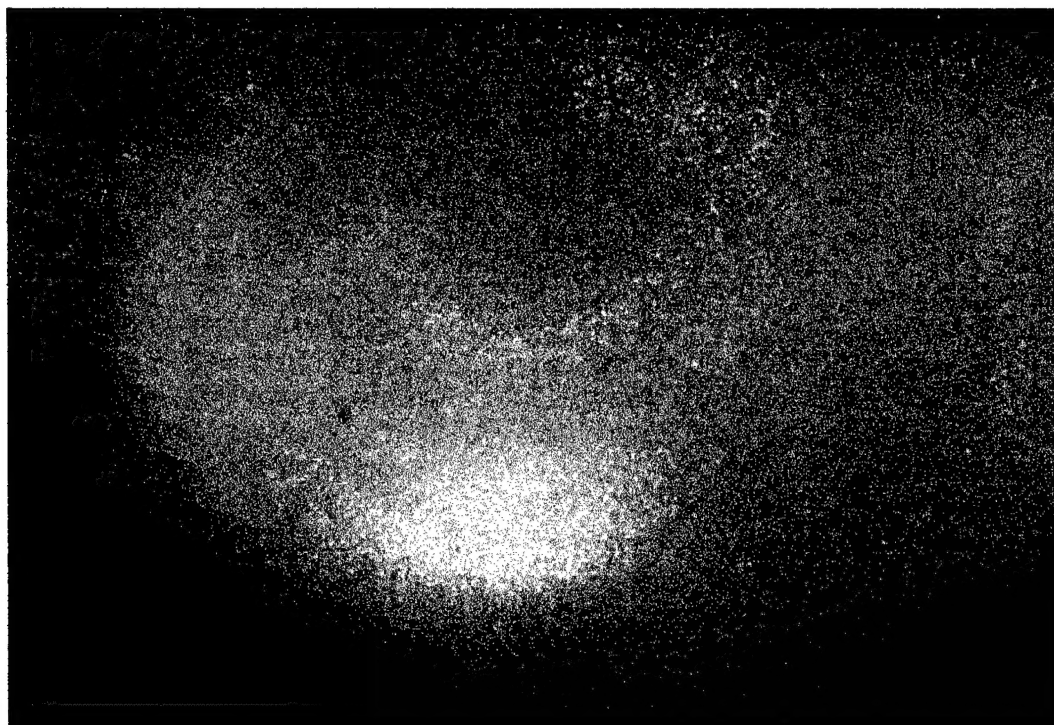
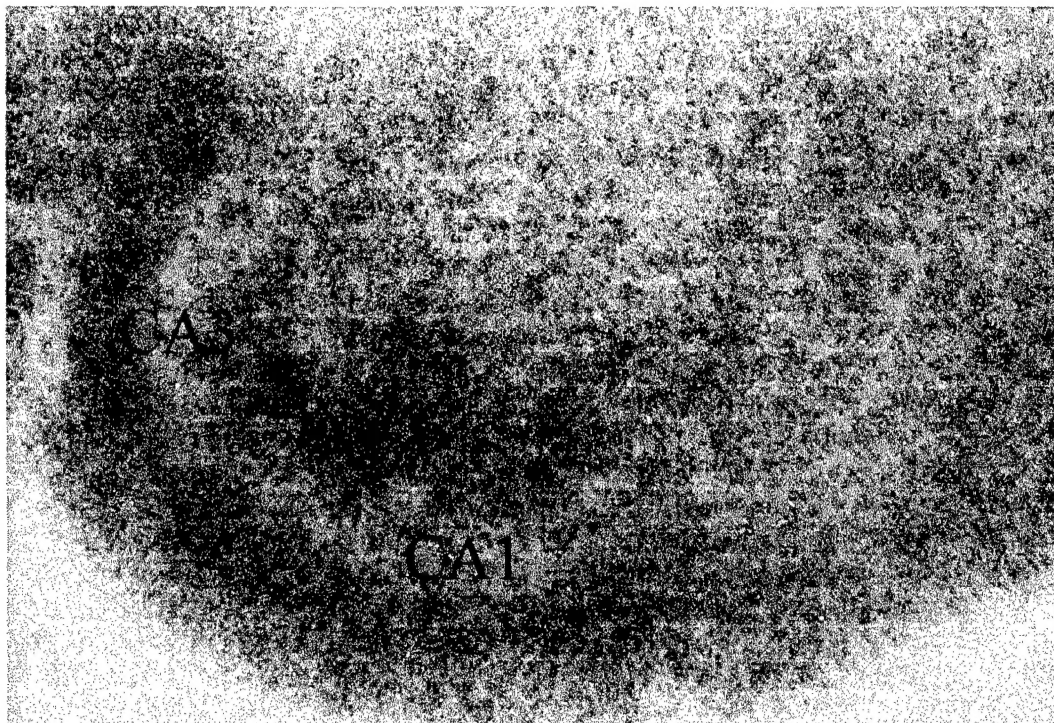


Figure 10

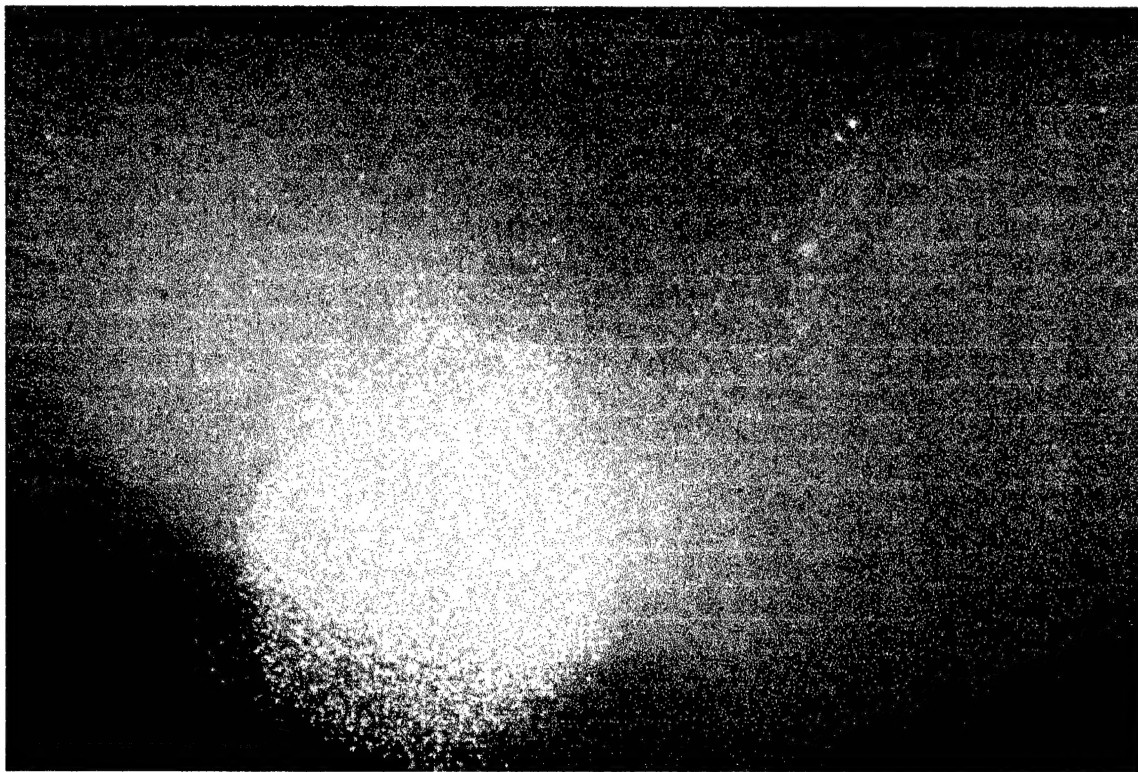
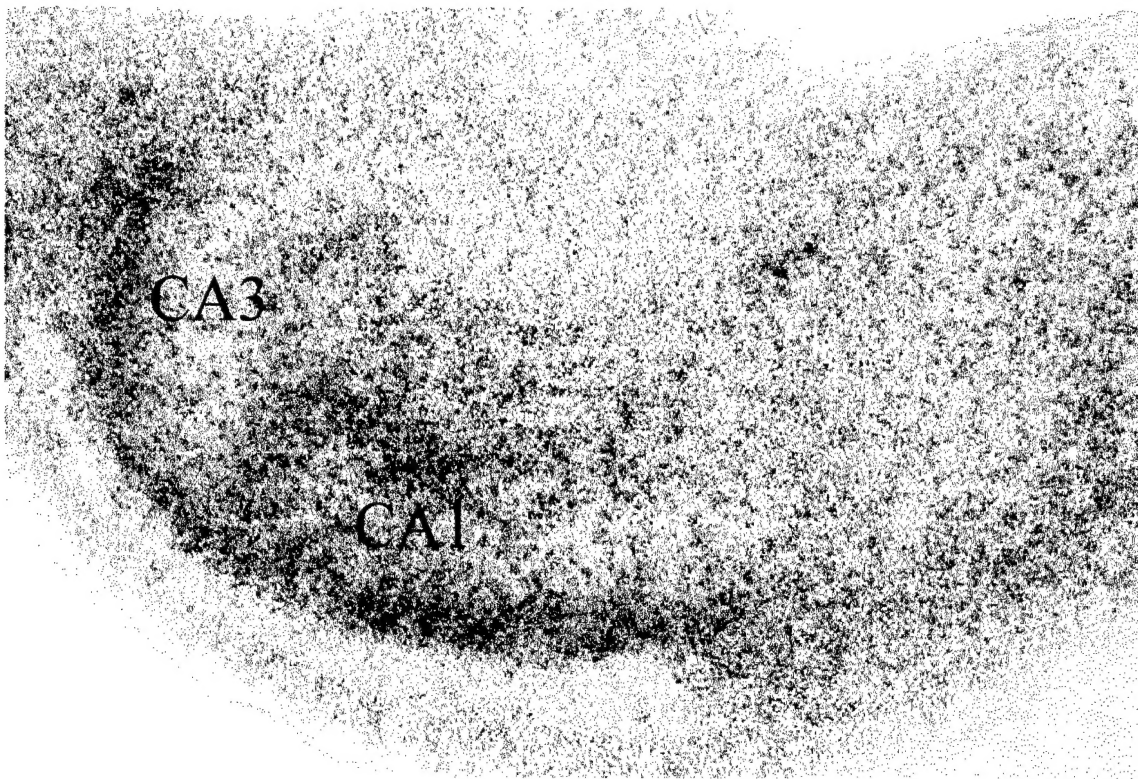


Figure 11